STUDIES ON THE PURIFICATION OF SQUID TYPE DFPASE SUITABLE FOR GENETIC ENGINEERING APPLICATION

STORAGE STABILITIES OF SQUID TYPE DFPASES FROM EAST COAST (ECHP) AND WEST COAST SQUID HEPATOPANCREAS (WCHP) TISSUES

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1. OBJECTIVE

As part of our efforts toward preparing and purifying the squid type DFPase to different levels of purification suitable for both the agent detoxification application and for the ultimate development of genetic methods of producing the enzyme, one of the major objectives of this research was to determine the stability characteristics of the squid type DFPase. On the basis of such a study, conditions for the storage and transport of this enzyme (both from WCHP and ECHP) for field use can be worked out.

2. Summary of the Work Performed on the Stability of DFPase

2.1 Stability of DFPase at two Levels of Purification

Effect of storage of the native enzyme preparations (initially filter-sterilized) at room temperature (15 to 30°C) and -20°C under ambient non-sterile conditions was examined with a view to develop conditions for their storage and field use. Filter-sterilized aliquots of the DFPases (P-30 gel-filtered and DEAE gradient-eluted fractions) were kept at room temperature and -20°C for 233 days and their activity measured initially every week and subsequently every two weeks. Results of the tests are shown in Tables 1 and 2.

The P-30 purified enzyme showed substantial loss of activity when kept in its native form at room temperature. Lyophilization resulted in 30-35% initial loss of activity. Despite the initial loss, lyophilization enabled both the P-30 purified enzymes retain 50-75% of their initial activity over a 233 day period. DFPase preprations from the East Coast Squid Hepatopancreas (ECHP) tissues retained relatively larger percent of their initial activity than those from the West Coast Squid Hepatopancreas (WCHP) tissues.

DEAE purified DFPases from both sources, viz., WCHP and ECHP, showed greater sensitivity to room temperature storage than their P-30 counterparts.

At -20°C the P-30 purified enzymes from both the sources, viz. WCHP and ECHP showed satisfactory stability, maintaining >90% retention over the test period of 233 days. At the level of DEAE purification the ECHP-enzyme appears to be relatively better than that from WCHP.

Besides the initial loss of activity due to lyophilization, both the ECHP and WCHP-enzymes appeared to incur very little loss over the 233 day period, with the ECHP enzyme exhibiting better activity retention than the WCHP. In this series of experiments, the samples were filter-sterilized at the outset. Subsequently during the storage, no sterile conditions were maintained.

In Appendix I are presented details of the experimental work and the results obtained from the 233-day long storage stability studies.

TABLE 1. STABILITY CHARACTERISTICS OF DFPASE: STORED AT ROOM TEMPERATURE

	P-30 % Retention	Days	DEAE % Retention	Days
Native Enzyme in BTP				
WCHP-DFPase	>50	100	>50	155
			~30	233
ECHP-DFPase	~50	30	~50	95
			v. low	233
Lyophilized and Stored				
WCHP-DFPase	~50	240	~100	60
			~50	85
			v. low	233
ECHP-DFPase	~70	240	~100	75
			~58	90

TABLE 2. STABILITY CHARACTERISTICS OF DFPASE: STORED AT -20°C

	P-30 % Retention	Days	DEAE % Retention	Days
Native Enzyme in BTP				
WCHP-DFPase	~100	60	Drops to 75	90
	>90	240	~75	233
ECHP-DFPase	~100	75	95-100	80
	95-100	240	~90	233
Lyophilized and Stored				
WCHP-DFPase	~100	80	~100	80
	~85-90	240	~90	233
ECHP-DFPase	100	80	~100	80
	>75	240	~90	233

APPENDIX I

STORAGE STABILITIES OF SQUID TYPE DFPASES FROM EAST COAST (ECHP) AND WEST COAST SQUID HEPATOPANCREAS (WCHP) TISSUES

Experiments were performed to determine the stabilities of the DFPase preparations obtained through the processing of West Coast Hepatopancreas (WCHP) and East Coast Hepatopancreas (ECHP) tissues.

In the first series of experiments whose results are presented in Table-A, material was taken directly from a P-30 column eluant and stored as single 1.5 mL aliquots. The observed increase in specific activity after 28 days of storage at 4°C is not as significant as indicated (Avg = 24%). This could be attributed to an observed fluctuation in the electrode calibration from its initial value. All other readings in this series were made under identical temperature, pH and standardization conditions. The data represented in Figure 1 show that WCHP P-30 active fractions appear to be more stable than ECHP P-30 fractions when stored under similar conditions. Bacterial contamination was noted after sometime. To avoid differences in the extent of contamination being a major cause of apparent stability differences, all samples for the second series of experiments (Tables B-1 and B-2 and the remaining figures) were filter-sterilized before further treatment and storage in individual aliquots. They were only opened on the day of testing. No attempt was made to put the enzyme in sterile containers as this would probably not be the way the enzyme would be treated in the field. Results obtained with P-30 gel-filtered samples stored at room temperature are given in Figure 2. Although bacterial growth was observed in the 28-day samples when they were plated on tryptic soy agar and incubated at 37°C overnight, bacteria were observed to be present in WCHP and ECHP P-30 samples to about the same extent.

TABLE A. STABILITY PROFILES OF ECHP-AND WCHP-DFPASES AT THE P-30 GEL FILTRATION LEVEL OF PURIFICATION

	DFPase Sp. Activity from ECHP-P-30			DFPase Sp. Activity from WCHP-P-30		
Number of days from start	Lower Sp. Activity	Actives	Highest Sp. Activity	Lower Sp. Activity	Actives	Highest Sp. Activity
Initial	2.94	3.79	4.01	4.16	4.31	4.45
28 days 4°C*	4.06	4.78	5.34	4.64	5.29	5.21
35 (RT)	3.50	4.45	5.00	3.95	4.75	4.55
42 (RT)	3.05	3.90	4.40	4.00	5.40	5.05
56 (RT)	1.65	2.60	2.62	3.35	5.00	4.70
70 (RT)	0.68	1.68	1.32	2.35	4.50	4.60
84 (RT)	0.26	1.00	0.96	1.80	3.90	4.10
98 (RT)	0.08	0.60	0.70	1.35	3.60	4.20
112 (RT)	0.04	0.40	0.48	0.80	3.05	3.50
126 (RT)	0.02	0.14	0.28	0.54	2.30	2.70
140 (RT)	0.01	0.04	0.08	0.41	1.25	2.30
154 (RT)	0.02	0.08	0.06	0.34	1.80	2.10
168 (RT)	0.00	0.00	0.05	0.15	1.10	1.30
182 (RT)	----	----	----	0.12	0.80	1.10
210 (RT)	----	----	----	0.07	0.52	0.68
246 (RT)	----	----	----	0.07	0.48	2.60
334 (RT)	----	----	----	0.01	0.12	0.21

*During the first 28 days after start, the samples were kept at 4°C after which they were kept at room temperature (RT).

TABLE B.1. STABILITY PROFILES OF ECHP-DFPASES AT ROOM TEMPERATURE AND AT -20°C

Number of days from start	DFPase Activity of P-30 Fraction				DFPase Activity of DEAE-Peak Fractions			
	Room Temperature		-20°C		Room Temperature		-20°C	
	Non-Lyoph	Lyoph	Non-Lyoph	Lyoph	Non-Lyoph	Lyoph	Non-Lyoph	Lyoph
Initial	6.00	5.20	5.8	5.20	13.5	11.0	14.00	11.00
7 days	5.60	4.00	5.40	5.20	15.0	7.0	13.00	9.00
21 days	3.30	3.60	5.40	5.80	15.0	8.50	14.00	14.00
35 days	1.20	3.70	5.50	5.70	14.50	6.50	13.60	10.00
49 days	0.30	3.90	5.20	5.60	15.50	7.00	13.00	11.50
63 days	0.10	3.80	5.20	5.50	8.40	6.50	13.50	11.00
77 days	0.07	3.60	5.20	5.80	9.00	7.00	14.00	10.00
91 days	0.05	2.80	4.00	4.60	8.00	4.20	9.50	9.00
119 days	0.10	2.60	3.60	4.20	6.00	4.20	11.50	9.00
147 days	0.05	1.60	3.20	3.40	3.10	2.40	12.00	7.60
233 days	0.00	2.60	3.40	4.80	1.97	0.25	12.00	11.20

TABLE B.2. STABILITY PROFILES OF WCHP-DEPASES AT ROOM TEMPERATURE AND AT -20°C

	Specific activity of P-30 Fraction				Specific activity of DEAE-Peak-1				Specific activity of DEAE-Peak-2			
Number of days from start	Room temp.		-20°C		Room temp.		-20°C		Room temp.		-20°C	
	Non-Lyo	Lyo.	Non-Lyo	Lyo	Non-Lyo	Lyo	Non-Lyo	Lyo	Non-Lyo	Lyo	Non-Lyo	Lyo
Initial	4.70	3.20	4.40	3.20	29.0	17.0	28.00	17.00	11.60	7.00	11.40	7.00
7 days	4.20	1.90	4.20	3.40	27.50	9.75	24.00	15.50	10.80	5.20	10.40	7.20
21 days	4.40	1.70	4.40	4.00	30.50	11.00	24.50	20.00	11.20	6.00	10.80	8.40
35 days	4.30	1.60	4.20	3.60	35.00	8.00	22.50	17.50	9.00	5.60	10.00	8.60
49 days	2.40	1.40	4.20	3.80	25.00	11.50	21.00	19.00	10.80	5.20	9.40	7.60
63 days	2.60	1.20	4.20	3.80	26.50	11.50	18.00	17.00	6.40	5.60	10.00	8.40
77 days	2.20	1.20	4.40	4.00	26.00	8.00	23.00	19.50	6.40	5.60	10.20	7.20
91 days	1.00	1.10	3.10	2.60	18.00	5.00	17.00	14.00	4.00	3.60	7.20	6.40
119 days	0.80	0.60	3.60	3.10	18.00	4.40	14.00	14.80	2.80	2.70	6.80	7.00
147 days	0.30	0.60	3.40	2.40	14.00	3.80	17.00	14.00	2.00	2.00	6.20	5.60
233 days	0.07	1.10	3.40	3.70	8.40	0.20	20.00	16.80	0.40	0.10	7.20	7.60

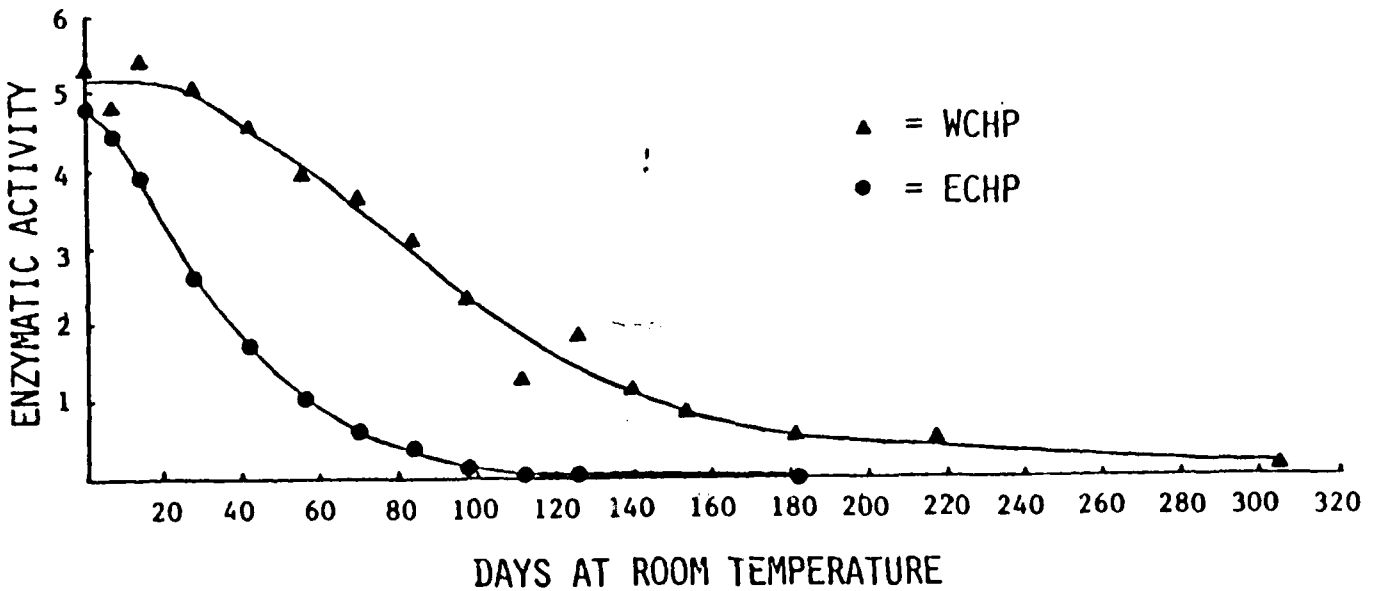

FIGURE 1. STABILITIES OF P-30 PURIFIED SQUID-TYPE DEPASES AT ROOM TEMPERATURE (15-30°C); INITIAL OBSERVATIONS.

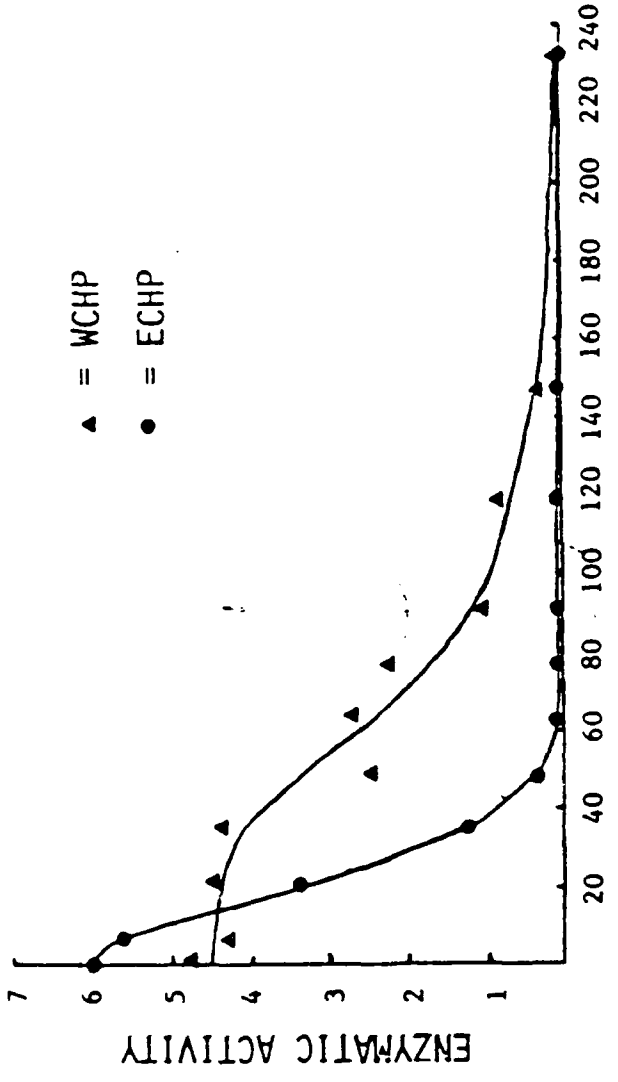

FIGURE 2. STABILITIES OF P-30 PURIFIED SQUID TYPE UREASES AT ROOM TEMPERATURE.

In the first series, the specific activity of the WCHP P-30 preparation was 3.85 units/mL vs 2.06 units/mL for the ECHP fractions. This added amount of protein in the ECHP preparation could be a source of additional proteases and bacterial nutrients promoting the observed stability differences. Sterile sample studies ans studies with protease inhibitor-treated vs non-treated samples will be undertaken to see if the ECHP sample is inherently less stable at room temperature or if it is merely more susceptible to bacterial contamination and proteolytic activity. What can be concluded from the data presented in figures 1 and 2 is that WCHP P-30 purified DFPase is more stable than ECHP P-30 purified material at room temperature under comparable non-sterile conditions.

Similar data are presented for DEAE-purified DFPase obtained from both sources (WCHP and ECHP) in Figure 3. Although the kinetics of activity loss do not seem to differ significantly, WCHP-Peak-1 retains 29% of it initial activity after 233 days at room temperature. However, WCHP Peak 2 sample retained 3.6% and ECHP-Peak-1, 13.1%. (Similar values for P-30 purified material are 1.4% for WCHP and 0% for ECHP.) The specific activities of the samples are: WCHP-Peak-1, 12.76; WCHP-Peak-2, 9.25 and ECHP-Peak-1, 3.93. No reasonable correlation between larger specific activities and increased stability was noted. But once again, one of the WCHP forms appears to have greater stability than the major ECHP-DEAE-purified form.

In a parellel series of experiments, both P-30 and DEAE-purified material were lyophilized and stored at room temperature. In each case, a large initial activity loss was noted as a result of lyophilization. The percent of the initial unlyophilized sample activity remaining after storage of the lyophilized sample at room temperature for 7 days is 66.7% for ECHP-P-30, 63.6% for ECHP Peak-1, 40.4% for WCHP P-30, 33.6% for WCHP Peak-1 and 34.7% for WCHP Peak-2. All the lyophilization graphs use a day-1 activity based on an essay of lyophilized sample immediately after it was lyophilized. This eliminates any effect due to the lyophilization itself from the effect due to storage at the specified temperature. The percentages calculated here take into account the effects due to both the lyophilization and storage in a lyophilized form by comparing the amount of activity remaining in the sample with the initial values of the unlyophilized samples. The P-30 samples (Figure 4) seem to reach a stable plateau.

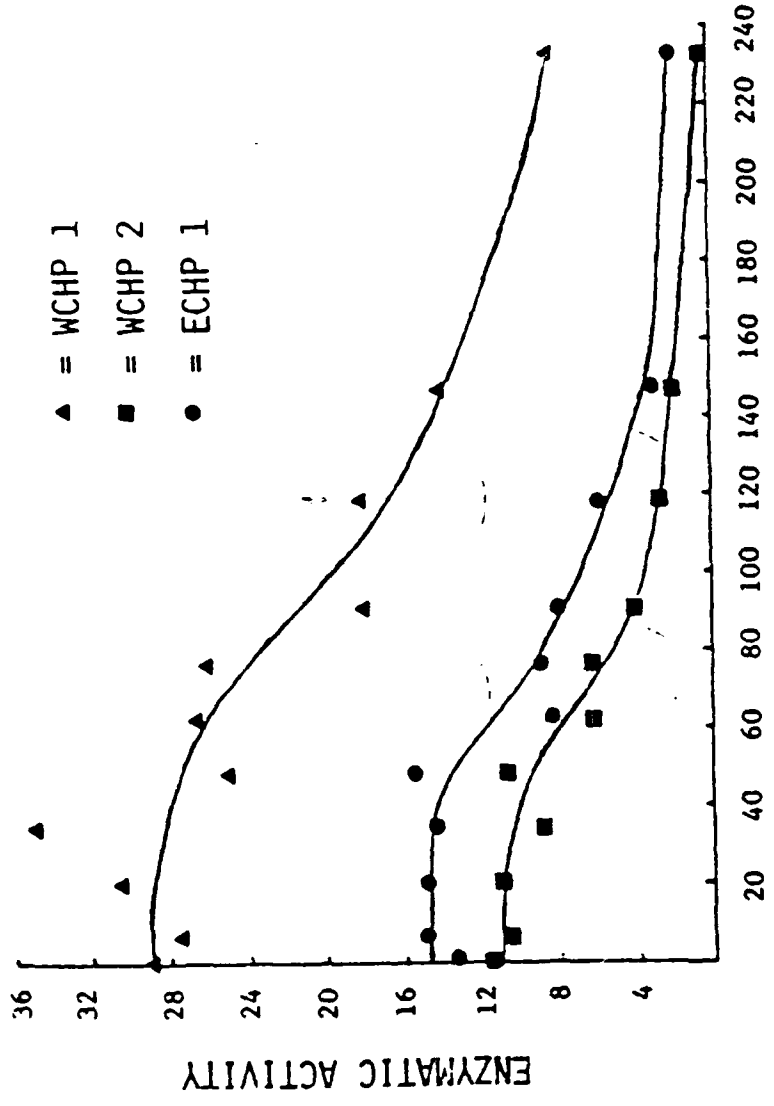

FIGURE 3. STABILITIES OF DEAE-PURIFIED SQUID TYPE DFPASES AT ROOM TEMPERATURE.

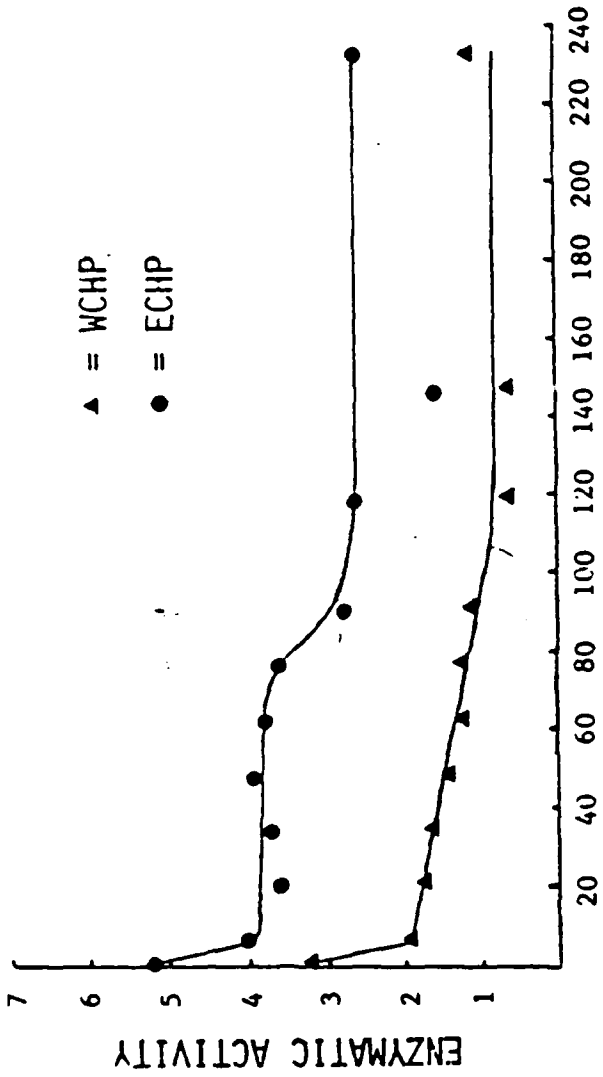

FIGURE 4. STABILITIES OF LYOPHILIZED P-30 PURIFIED SQUIDE-TYPE DPASE AT ROOM TEMPERATURE.

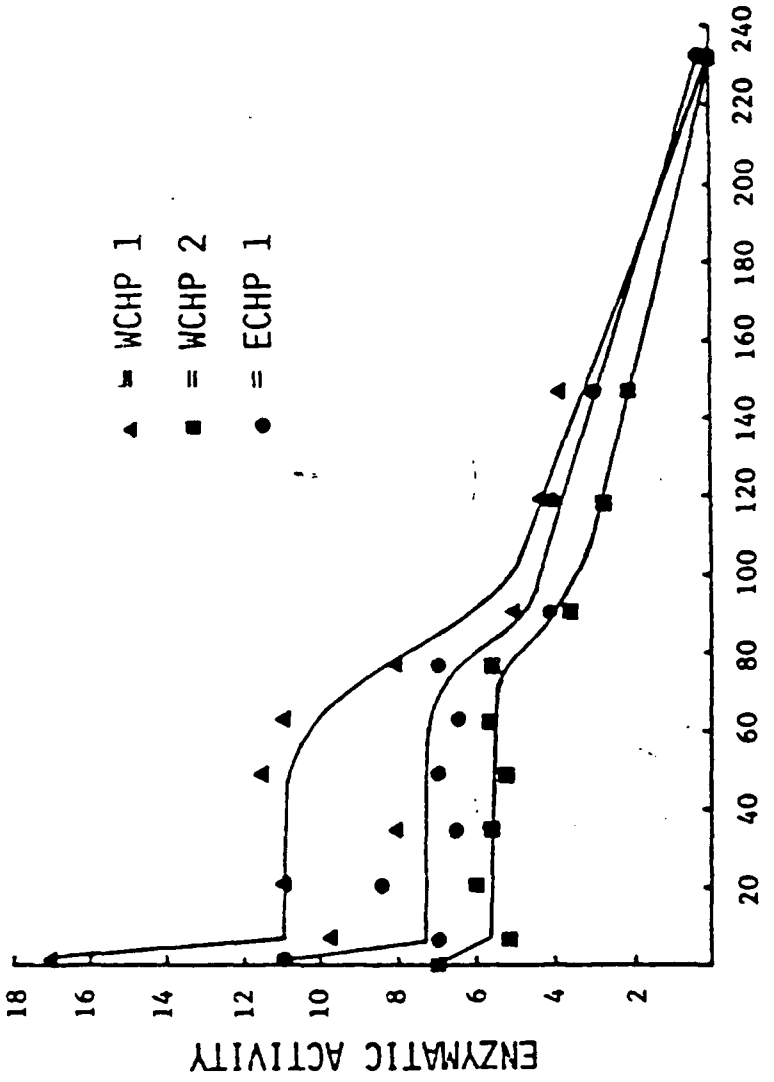

FIGURE 5. STABILITIES OF LYOPHILIZED DEAE-PURIFIED SQUID-TYPE DFPASE AT ROOM TEMPERATURE.

The ECHP P-30 sample retains 43.3% of its initial activity after 233 days at room temperature, the WCHP P-30 sample retains 18.9% of its initial activity. Data from Figure 5 shows that DEAE DFPase preparations may be stable for the first 77 days but they eventually lose all but an insignificant amount of activity by day 233. Despite the initial activity loss, lypholization may allow long term storage of P-30 purified DFPases at room temperature, with ECHP retaining a higher percentage of the initial activity.

In a parallel (second) series of experiments, identical sets of samples were stored at -20°C. These samples were all found to be more stable than their room temperature counterparts. While investigating the stability, a problem alluded to earlier was once again observed. From day 1 to day 77 (in the 233-day storage study), the F^- electrode was standarized at 31°C with 2×10^{-5} and 2×10^{-4} M F^- in 5 mM Bis-Tris Propane, pH 7.2, 400 mM KCl, 50 mM NaCl. A new standard was made for day 91, the only difference being that the buffer was now 10 mM Bistris propane, pH=7.0, 400 mM KCl, 50 mM NaCl. Since there was an average of 27% decrease in the measured activity on this day and since this lower value was retained on days-119 and 147 by samples which had previous to day-91 been stable at a higher value, selected 147-day samples were tested at days-158 and 159 (interium storage at 4°C) with the initial pH 7.2 on day 158 and the new pH 7.0 standard on day 159. (An additional precaution undertaken in this connection was to use a new electrode. However, it was later shown to give the same results as the one used previously). Assays were always done at 30°C in 400 mM KCl, 50 m NaCl, 10 mM Bistrispropane, pH=7.0. These data are shown in Table C.

TABLE C. EFFECT OF CALIBRATION MEDIA PH'S ON ENZYME ACTIVITY

Sample	147 days pH = 7.0 Standard	158 days pH = 7.2 Standard	159 days pH = 7.0 Standard	Avg of 3	% Decrease Day 158 to 159
ECHP DEAE Lyopho, R.T	2.40	3.20	3.20	2.93	0
WCHP DEAE 2 Lyopho, R.T	2.00	2.60	2.40	2.33	8
ECHP DEAE Lyopho, -20°C	7.60	9.60	8.80	8.66	8
WCHP DEAE 1 Lyopho, -20°C	14.00	17.00	14.00	15.00	18
WCHP DEAE 2 Lypho, -20°C	5.60	7.20	5.60	6.13	22
Slope on meter	55.5	58	55.7	----	----

The averages of three determinations (see Table-C) are presented in the graphs of this data rather than the numbers in Tables B-1 and B-2.

Further data which point to the relatively high degree of variability in results which can be attributed to small differences in standardization procedure are shown in Table-D. The 2 x 10^{-5} M F^- standard was made to have several millivolt readings. The variations in the test data were within the variations that had been observed for that standard both upon comparison with other 2 x 10^{-5} F^- standards and reading obtained with the same standard at different times. For a given 2 x 10^{-5}m F^- millivolt reading, the slope was varied within the range it is known to have varied during the course of these experiments. The same sample was assayed twice under each condition. Both the actual data and an average values are reported (units-change in F^- molarity/0.5 min). The results given in Tables A and B are also the results of similarly precise duplicate assays but only the average is given.

TABLE D. EFFECT OF VARIATION IN ELECTRODE CALIBRATION ON THE ACTIVITY OF THE ENZYME SAMPLES

2 x 10^{-5} MF^- (mV)	116.9		115.0		113.0	
Slope = 55.0	0.461		0.458		0.424	
	0.490	0.476	0.453	0.456	0.425	0.424
58.6	0.435		0.408		0.351	
	0.457	0.446	0.405	0.406	0.359	0.355
60.0	0.421		0.387		0.331	
	0.411	0.416	0.375	0.381	0.334	0.332

The average % decrease between the first number and the last number in a row or column was 17.0 ± 4.5%. In order to prevent problems with data variability due to changes in the meter standarization conditions, we have determined that a 2 x 10^{-5} M F^- standard (400 mM KCl, 50 mM NaCl, 5 mM BTP, pH = 7.2, F^- diluted from a 0.1 M Orion stock) that gives a mV reading of 117.9 will give a slope = 60.15, the theoretical value fo the slope at 30°C. When the 2 x 10^{-4} M F^- standard from which it was made is used to complete the calibration. Future stability studies will be made on a meter where the meter reading in
2 x 10^{-5} MF^- is set to 117.9 mV and the slope is set to 60.15. Periodic checks

will be made of the concentration value obtained for the 2×10^{-4} M F^- standard to detect any possible electrode failure. For the current series of assays, the assays on day-233 were done with the meter calibrated with the pH = 7.2 standard used before day-91. In cases where it was warranted, the mean activity for days up to 77 was calculated and compared to the mean for days 91-223. If the percent decrease in the mean activity for the later days was 22% or less the data was plotted two ways: first with a line through the individual means and then with a straight line through the mean of all the numbers. These latter graphs are made to suggest that any apparent activity loss indicated in the companion figure may be due to inherent assay variability and not due to any real activity loss. (Note the dotted line in Figure 9 reflects a 26% difference in the means which probably does indicate an activity loss).

With these parameters in mind, the stability profiles in Figures 6 and 7 may be taken to indicate that WCHP and ECHP P-30 material both reach stable plateus at -20°C, ECHP retaining 66% of its initial activity at 233 days; WCHP retaining 79% which may be interpretted to indicate it has not lost any activity (Figure 7). These results are probably too similar to say anything conclusive about relative superiority of WCHP-versus-ECHP-P-30 DFPase stability at -20°C.

At the level of DEAE-purified samples (Figure 8), the relative stability trends for the P-30 purified material are essentially reversed. ECHP Peak 1 retains 83% of its initial activity at 233 days (possibly insignificant, See Figure 9); WCHP Peak-2, 66% of its initial value (if 27 units/mL is considered the initial value, See Figure 8); and WCHP Peak 2, 66% of its initial value. The exact reason why this occurs is not known especially given the reverse trend when DEAE material is stored at room temperature (WCHP Peak 1 material retains more activity than ECHP Peak 1 material at room temperature). Taken together, these trends could point to a greater susceptibility of DEAE purified WCHP-DFPase to activity loss due to freeze-thawing (DEAE preparations are also stored at a higher ionic strength, we are not sure what effect that could have). There may be a need for the addition of stabilizing proteins such as BSA when WCHP samples are frozen. Data to substantiate are probably needed.

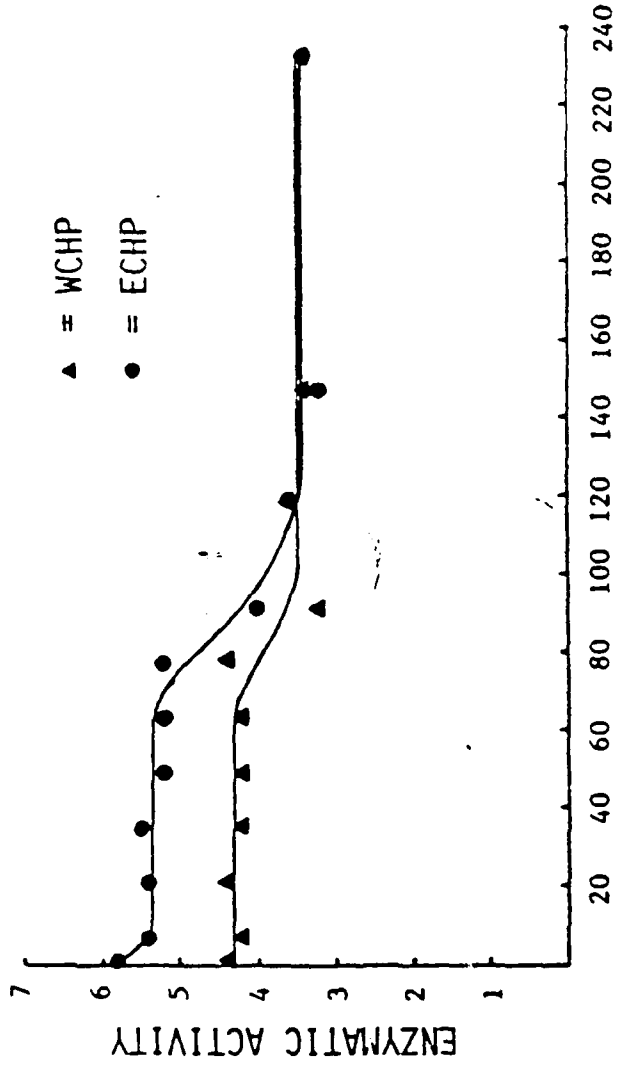

FIGURE 6. STABILITIES OF P-30 PURIFIED SQUID-TYPE ESTERASES AT -20°C.

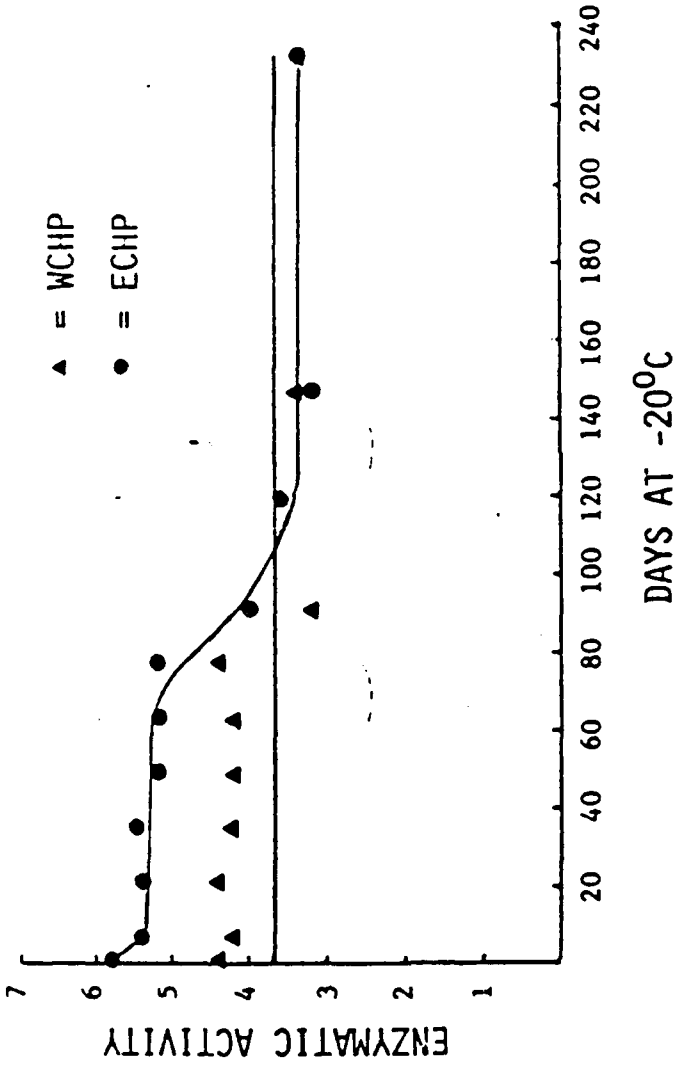

FIGURE 7. STABILITIES OF P-30 PURIFIED SQUID OFPASE AT -20°C ALLOWING FOR INHERENT ASSAY VARIABILITY BY F⁻ ELECTRODE METHOD.

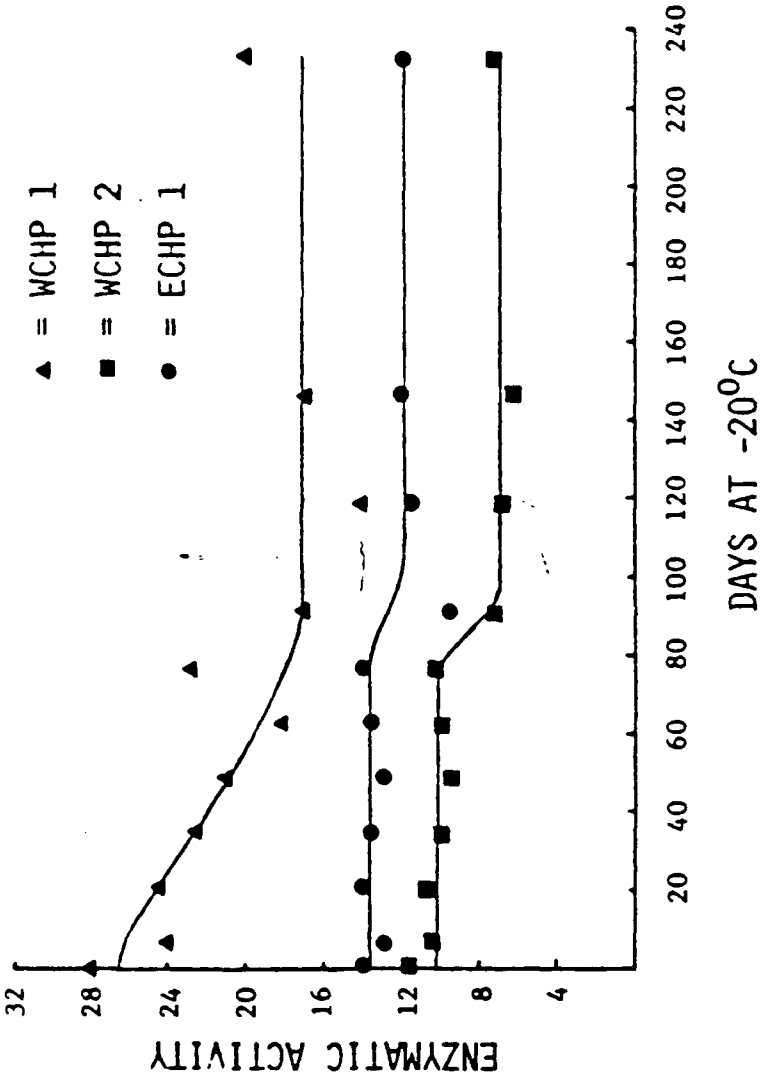

FIGURE 8. STABILITIES OF DEAE-PURIFIED SQUID-TYPE ATPASES AT -20°C.

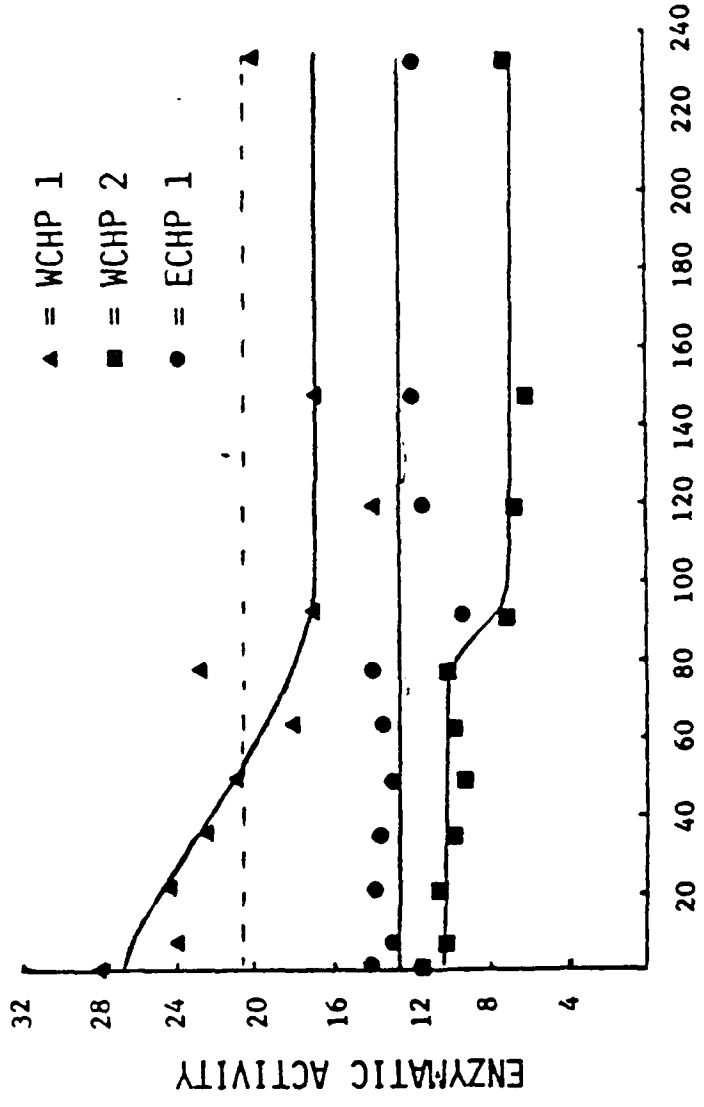

FIGURE 9. STABILITIES OF DEAE-PURIFIED SQUID-TYPE DFPASES AT -20°C ALLOWING FOR INHERENT ASSAY VARIABILITY BY THE F- METHOD.

The initial losses in activity when P-30 and DEAE lyophilized material are stored at -20°C are much smaller than for those stored at room temperature. In the case of the lyophilized samples stored at room temperature, there is an activity loss due to storage in addition to the initial loss caused by lyophilization. In the case of -20°C-stored material, all activity loss could be due to lyophilization alone. With the exception of the non-lyophilized WCHP DEAE Peak 1 sample where variability indicates that 27 units/mL should be used as an initial value, one can judge the effect of lyophilization by comparing the mean value of assays from 1-77 days for lyophilized samples (Figure 10 and 12) to those for unlyophilized samples (Figure 6 and 8) the percent activity remaining is 102% for ECHP P-30, 80.1% for ECHP Peak 1; 86.0% for WCHP P-30; 66.2% for WCHP DEAE Peak 1, and 75.7% for WCHP Peak 2. ECHP once again appears to be more stable to lyophilization. Lyophilized P-30 samples stored at room temperature retained more activity in the long run. With one exception, the lyophilized samples stored at -20°C still had less activity than their unlyophilized counterparts at 233 days storage if one uses the mean of the values at 91-233 days as a measure of the stability; the average value of the lyophnilized samples is 120% of that for the unlyophilized samples for ECHP P-30, 84.2% for ECHP Peak 1, 87.4% for WCHP P-30, 89.1% for WCHP Peak 1 and 99.0% for WCHP Peak 2. These differences are fairly insignificant and point to the fact that all samples could be stored in a lyophilized condition at -20°C, with the possible exception of the ECHP P-30 purified samples. There seems little reason to do so. The activity losses in the lyophilized samples that occur after 71 days are also probably not significant as indicated in Figures 11 and 13.

Figures 14-18 merely illustrate some of the conclusions from the data presented here. The dramatic increases in activity loss at room temperature, the stabilizing influence of Lyophilization on the room temperature ECHP P-30 and to a less significant extent the WCHP P-30 samples despite large initial activity losses, the stability of ECHP Peak-1 material at -20°C and the relative ineffectuality of Lyophilization to provide any enhanced stabilization for storage at -20°C (the latter two shown more clearly if longer times had been presented). Important conclusions are:(i) lyophilization is a means of ultimate P-30 sample stabilization and(ii) there is a need to find a way, if possible, to prevent the initial WCHP Peak-1 and-2 activity losses at -20°C.

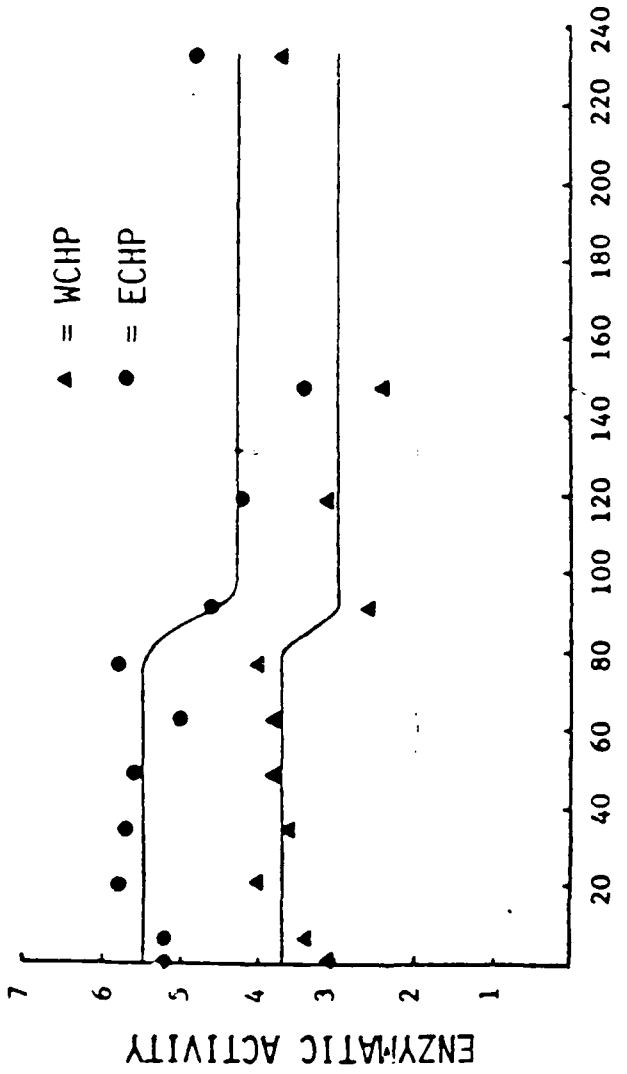

FIGURE 10. STABILITIES OF LYOPHILIZED P-30 PURIFIED SQUID-TYPE DEPASES AT -20°C

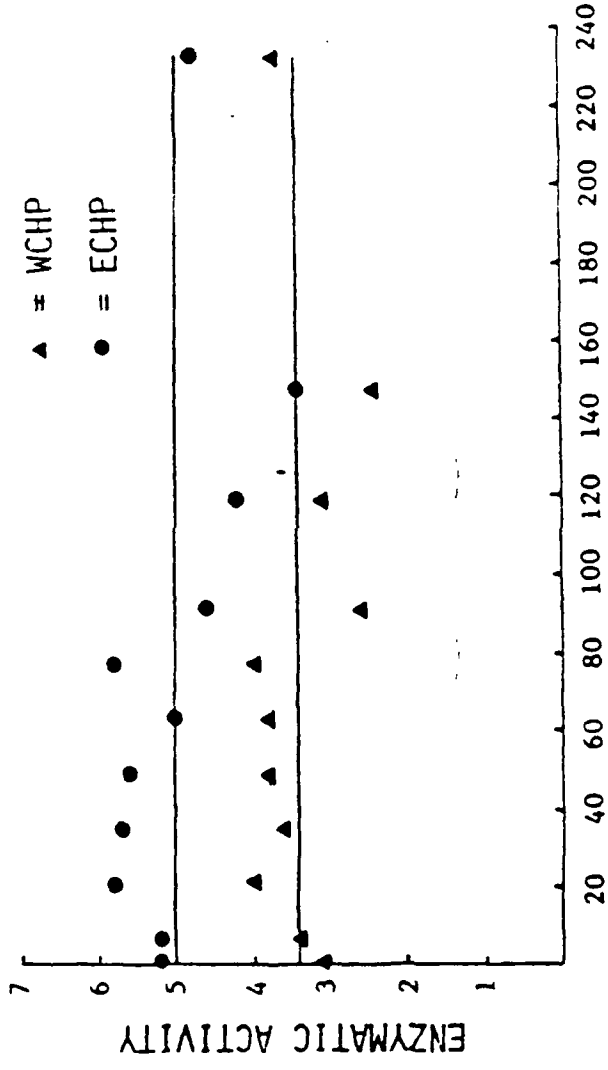

FIGURE 11. STABILITIES OF LYOPHILIZED P-30 PURIFIED SQUID-TYPE ESTERASES AT -20°C ALLOWING FOR INHERENT ASSAY VARIABILITY BY THE F⁻ ELECTRODE METHOD.

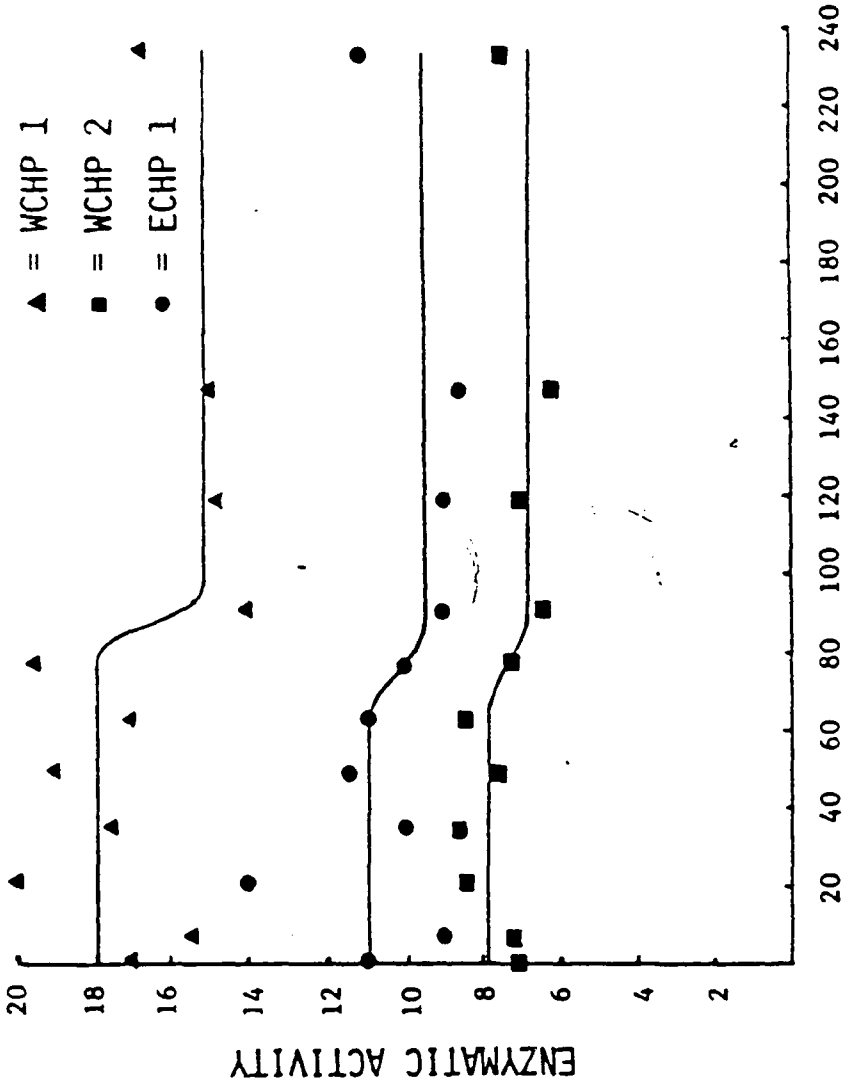

FIGURE 12. STABILITIES OF LYOPHILIZED DEAE-PURIFIED SQUID-TYPE DIPASES AT -20°C

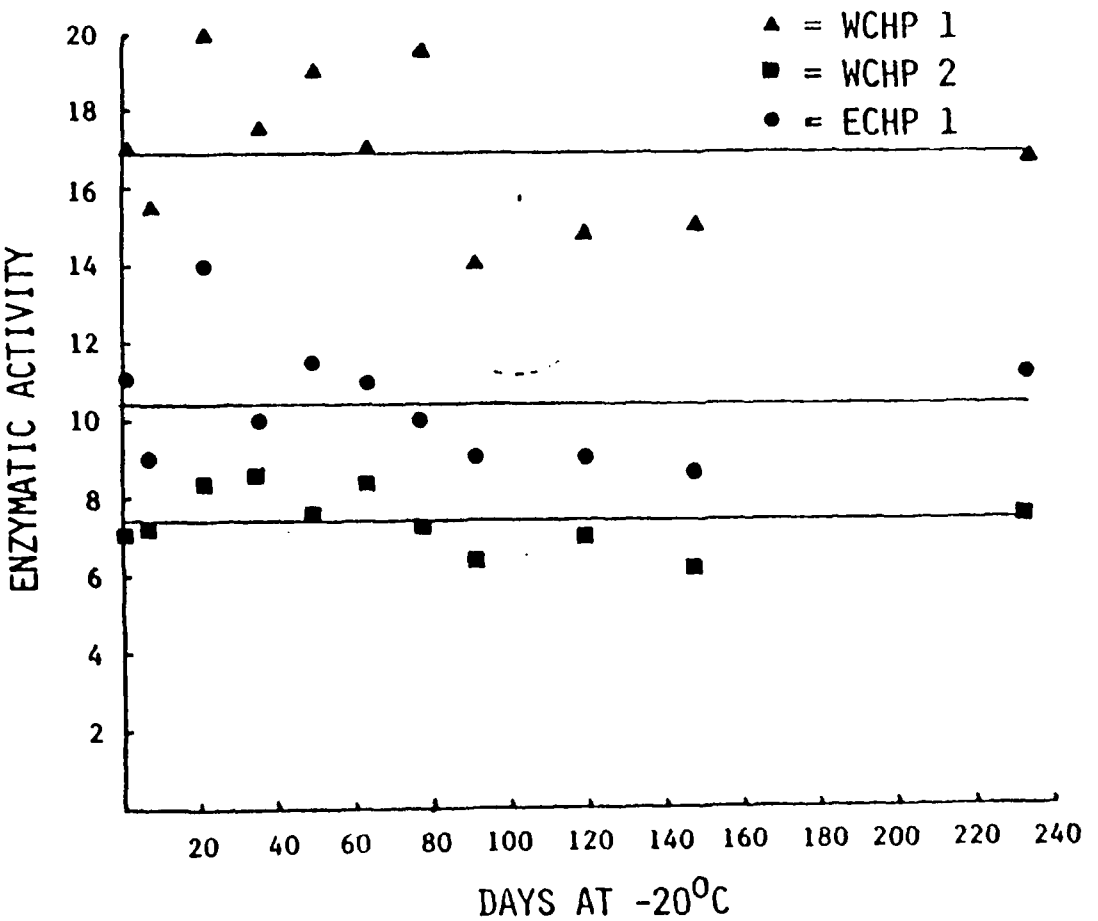

FIGURE 13. STABILITIES OF LYOPHILIZED DEAE-PURIFIED SQUID-TYPE DEPASES AT -20°C ALLOWING FOR INHERENT ASSAY VARIABILITY BY THE E- ELECTRODE METHOD.

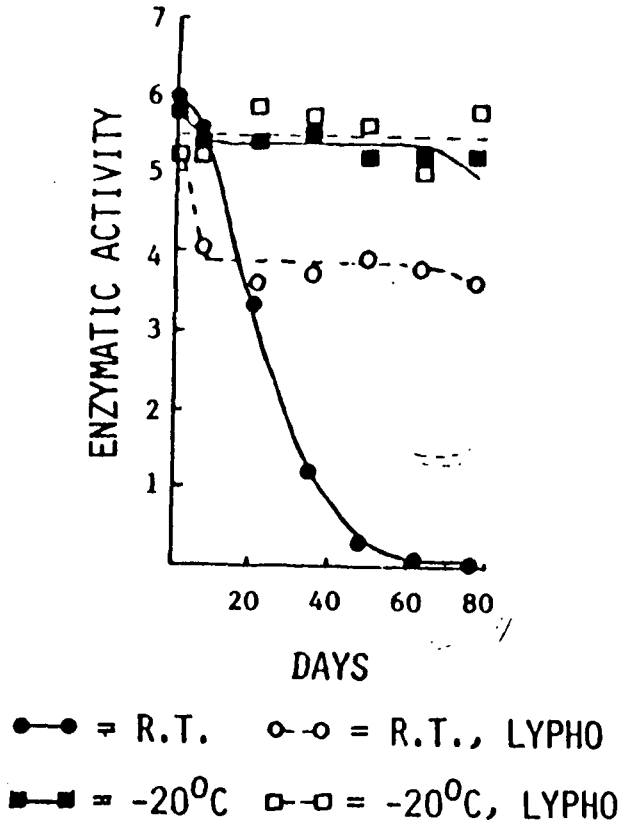

FIGURE 14. OVERVIEW OF THE STABILITIES OF P-30-PURIFIED ECHP-DEPASE UNDER DIFFERENT CONDITIONS.

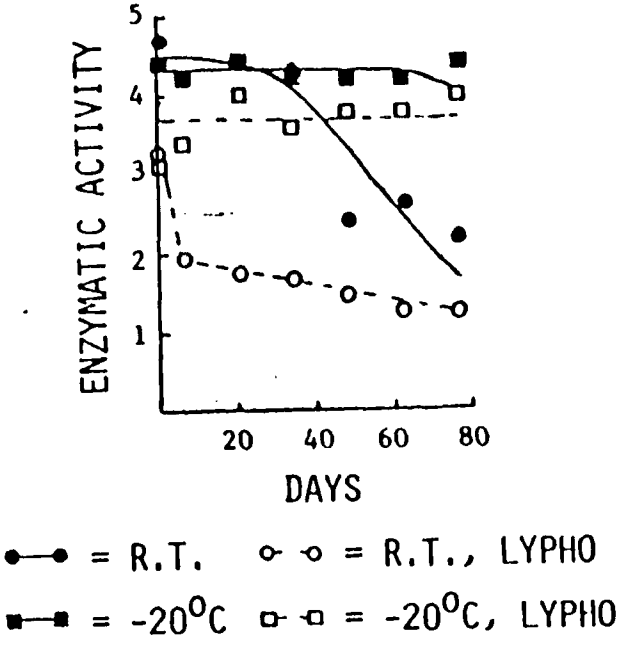

FIGURE 15. OVERVIEW OF THE STABILITIES OF P-30-PURIFIED WCHP-DEPASE UNDER DIFFERENT CONDITIONS.

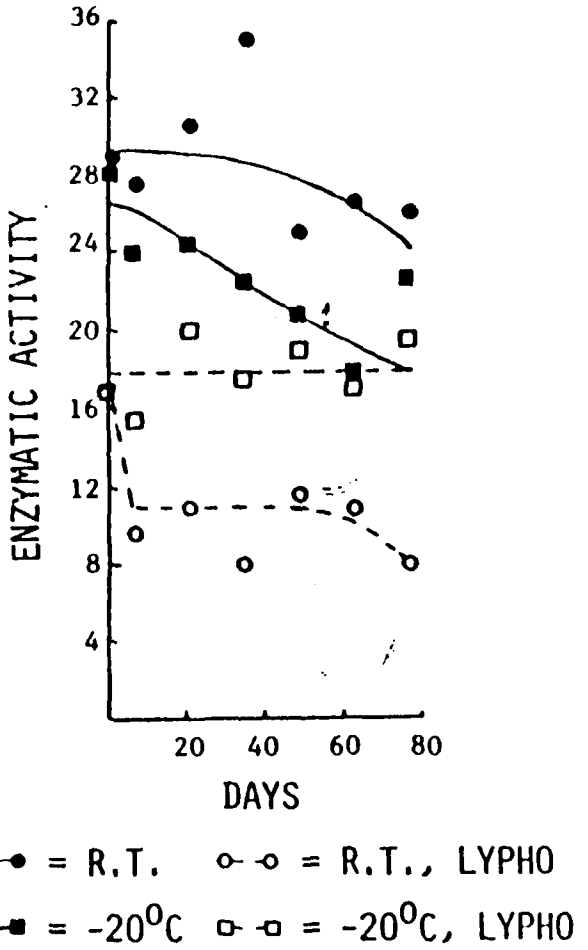

FIGURE 16. OVERVIEW OF THE STABILITIES OF DEAE-PURIFIED (WCHP) DFPASE, PEAK-1 FRACTION.

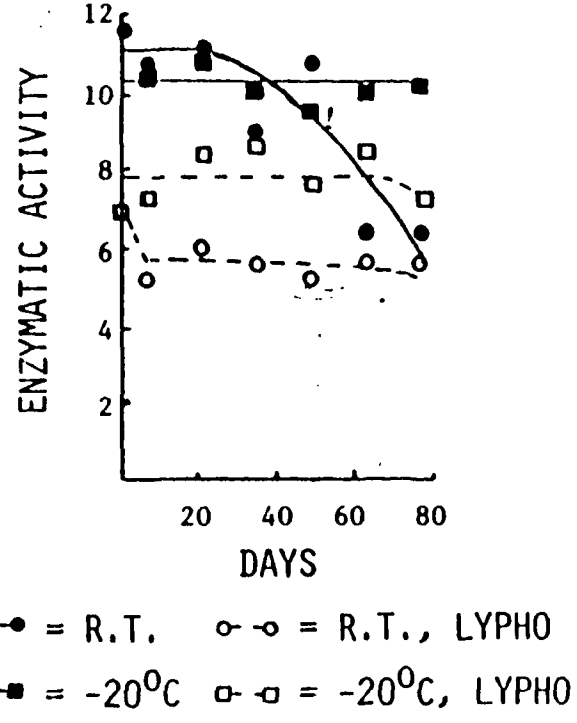

FIGURE 17. OVERVIEW OF THE STABILITIES OF DEAE-PURIFIED (WCHP) DFPASE, PEAK-2 FRACTION.

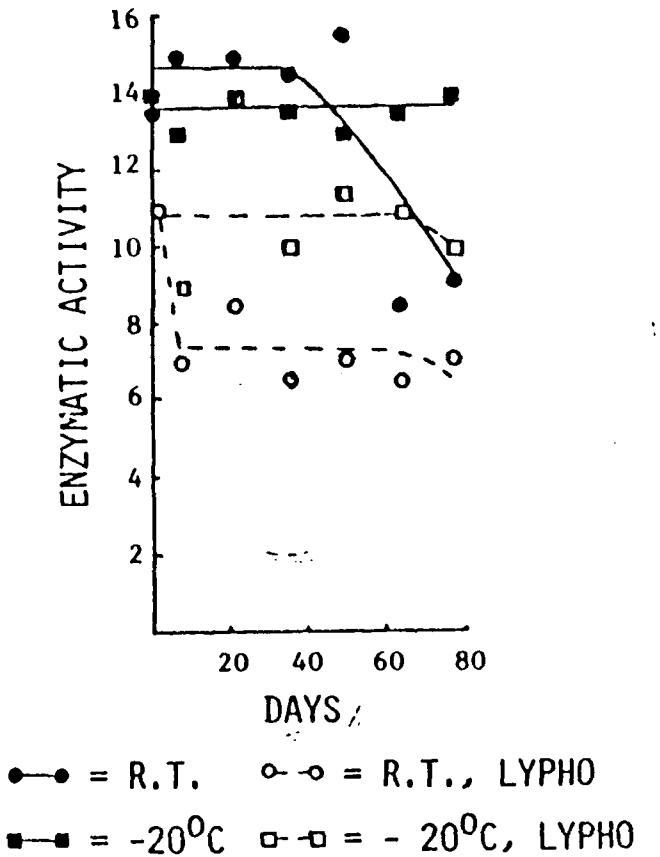

FIGURE 18. OVERVIEW OF THE STABILITIES OF DEAE-PURIFIED ECHP-DEPASE PEAK-1 FRACTION.